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Life, Death, and Ubiquitin: Taming the Mule

Ubiquitin-mediated protein degradation is an efficient way for the cell to get rid of unwanted proteins. A key player in this process is the E3 ubiquitin ligase. In this issue of *Cell*, Chen et al. (2005) and Zhong et al. (2005) describe a new E3 ligase, ARF-BP1/Mule, which targets two very different substrates, p53 and Mcl-1, with completely different cellular outcomes.

Last year, Hershko, Ciechanover, and Rose were awarded the Nobel Prize in Chemistry for the discovery of ubiquitin-mediated protein degradation. In this tightly regulated process, proteins are tagged with ubiquitin moieties through a series of enzymatic reactions involving an E1-activating enzyme, an E2-conjugating enzyme, and an E3 ubiquitin ligase. Tagged proteins are then degraded by the 26S proteasome (Hershko and Ciechanover, 1998). The E3 ligases are the "brain" of this process and determine substrate specificity. Hence, they are attractive candidates for therapeutic targets. In the current issue of *Cell*, Chen et al. (2005) and Zhong et al. (2005) describe a new E3 ligase, ARF-BP1/Mule, a gigantic (nearly 500 kDa) protein that possesses some unexpected structural features and mediates complex biological effects.

The two groups identified ARF-BP1/Mule by taking very different approaches. Chen and colleagues (Chen et al., 2005) set out to identify new regulators of the p53 tumor suppressor network. Following oncogenic and genotoxic stress signals, p53 is stabilized and biochemically activated, leading to transcriptional upregulation or repression of a multitude of target genes (Oren, 2003). Transcriptional alterations mediated by p53 result in a variety of cell fate changes, including growth arrest and apoptosis. This serves to prevent damaged cells from giving rise to progeny with defective genomes that eventually may spawn formation of tumors. Normally, the cell maintains low levels of p53 through rapid ubiquitin-dependent proteolysis of this tumor suppressor protein. Several E3 ligases have been implicated in this process, including the major negative regulator of p53, Mdm2 (Oren, 2003), as well as the more recently described COP1 and PIRH2 (Dornan et al., 2004; Leng et al., 2003). Another important regulator of p53 stability is the tumor suppressor protein ARF (Lowe and Sherr, 2003). Oncogenic stress induces expression of ARF, which then binds to Mdm2 in the cell nucleus or nucleolus and blocks the E3 activity of Mdm2, promoting p53 stabilization (see Figure 1). However, ARF can also operate in an Mdm2- and p53-independent manner (Lowe and Sherr, 2003). Thus, overexpression of ARF inhibits the proliferation of cells that lack both p53 and Mdm2, and deletion of ARF results in a wider range of tumor types and accelerated tumor formation in double null mice lacking both p53 and Mdm2. Hence, ARF probably targets other proteins in addition to Mdm2.

In their search for ARF binding proteins that are likely to underlie the p53-independent effects of ARF, Chen et al. (2005) came up with a new protein that interacts with ARF, which they dubbed ARF-BP1 (binding protein 1). From its sequence, the authors deduced that this protein is a novel E3 ligase. Indeed, ARF-BP1 was shown to possess E3 activity, which was inhibited by ARF. Fulfilling their initial suspicions, they were able to mimic faithfully the effects of ARF overexpression in p53 null cells using RNAi-mediated ablation of ARF-BP1, resulting in growth inhibition. Hence, the p53-independent antiproliferative effects of ARF can be attributed at least in part to the ability of ARF to neutralize ARF-BP1. Surprisingly, when p53 was present, ARF-BP1 ablation also resulted in stabilization and activation of p53. Further analysis revealed that ARF-BP1 binds directly to p53 and ubiquitylates this tumor suppressor, firmly establishing ARF-BP1 as an additional E3 ligase that targets p53. Remarkably, the biological outcome of ARF-BP1 ablation in p53-positive cells was enhancement of p53-mediated apoptosis.

Apoptosis is a highly regulated active program of cell death that is crucial for normal development and tissue homeostasis. Compromised apoptosis, or conversely excessive apoptosis, results in many human diseases, including cancer and neurodegenerative diseases. Proteins of the Bcl-2 family are lead players in apoptosis and are roughly divided into proapoptotic and antiapoptotic members. In response to various stress signals, proapoptotic members of the Bcl-2 family enable the release from mitochondria of apoptogenic factors such as cytochrome c and Smac/DIABLO. This leads to activation of caspases, which are proteases that cleave specific proteins and induce the morphological and biochemical features of apoptosis.

One prosurvival member of the Bcl-2 family, Mcl-1, is important for development and for responses to environmental cues (Michels et al., 2005). Unlike most other members of the family, Mcl-1 is a short-lived protein, the expression and antiapoptotic activity of which is normally controlled by the ubiquitin/proteasome pathway. Using a classical biochemical fractionation approach, Zhong et al. (2005) set out to identify the E3 ligase responsible for Mcl-1 ubiquitylation and degradation. The quest yielded a new E3 ligase, which they called Mule (short for Mcl-1 ubiquitin ligase E3). In addition to containing a HECT domain required for its E3 activity, Mule also harbors a BH3 (Bcl-2 homology 3) domain. BH3 domains mediate protein-protein interactions within the Bcl-2 family; indeed, the BH3 domain of Mule is necessary for its specific association with its substrate, Mcl-1. Downregulation of Mule expression resulted in stabilization and accumulation of Mcl-1; in line with the known antiapoptotic effects of Mcl-1, this rendered the cells more resistant to killing by genotoxic agents. Unexpectedly, Mule turns out to be identical to ARF-BP1. ARF-BP1/Mule thus regulates the stability of two very different proteins, p53 and Mcl-1.

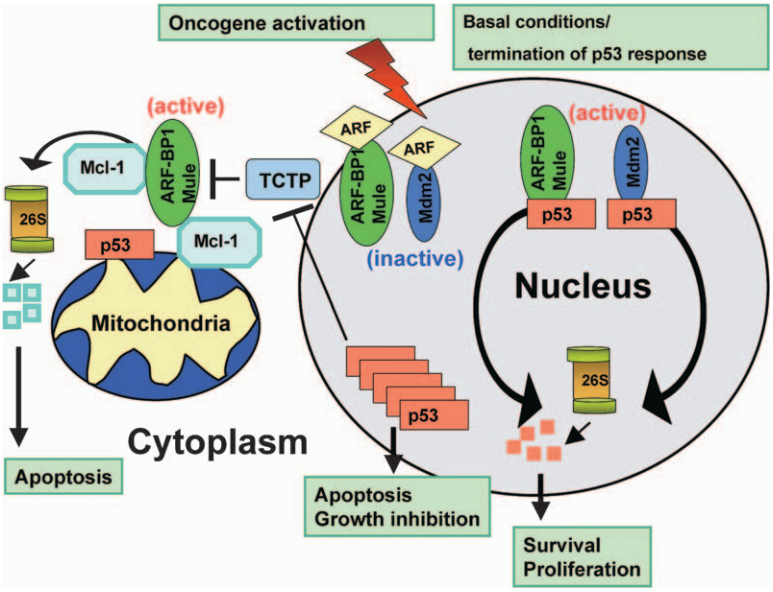

Figure 1. Proposed Mode of Action of ARF-BP1/Mule

When the cell is under basal nonstress conditions or when the p53 response is terminated, p53 is efficiently ubiquitylated by both Mdm2 and ARF-BP1/Mule, resulting in its rapid degradation. Oncogene activation leads to upregulation of ARF expression and results in binding of ARF to both Mdm2 and ARF-BP1/Mule. This results in blocking the nuclear activity of ARF-BP1/Mule, an E3 ligase. Inactivation of this E3 ligase causes the accumulation of p53, leading to apoptosis or growth inhibition. On the other hand, ARF-BP1/Mule in the cytoplasm ubiquitylates Mcl-1 and promotes its degradation. In this case, ARF-BP1/Mule activity, rather than inactivity, facilitates apoptosis. The interaction of ARF-BP1/Mule with Mcl-1 may occur in the cytoplasm as well as in mitochondria. p53 can translocate to mitochondria in some apoptotic scenarios. This translocation, as well as the fate of mitochondrial p53, may also be subject to regulation by ARF-BP1/Mule. TCTP, whose expression is repressed by p53, can prevent Mcl-1 ubiquitylation, perhaps by counteracting the activity of ARF-BP1/Mule. "Active" and "inactive" relate to the activity state of the E3 ligases ARF-BP1/Mule and Mdm2.

Comparison of the findings reported by Zhong et al. (2005) and Chen et al. (2005) reveals that the biological implications of the two studies appear to differ widely. Whereas Chen et al. assign to ARF-BP1/Mule an antiapoptotic function (via p53 degradation), Zhong et al. conclude that it actually promotes apoptosis (via Mcl-1 degradation). This is most vividly illustrated by the seemingly contradictory outcomes of ARF-BP1/Mule RNAi-mediated knockdown: reduced survival of p53-positive cells in one study (Chen et al., 2005) versus increased survival in the other study (Zhong et al., 2005). The fact that both groups used exactly the same cell line (U2OS) makes this picture even more enigmatic. One must assume that the biological impact of ARF-BP1/Mule is regulated in subtle ways, leading to very different outcomes even under superficially similar conditions. Relative availability of growth/survival factors, differences in cell-cell interactions, and different types of stress inadvertently inflicted on the cells may provide at least a partial explanation for this apparent inconsistency.

How does one incorporate the divergent effects of ARF-BP1/Mule into a coherent picture? One possibility is that, under normal conditions, ARF-BP1/Mule helps to maintain homeostatic levels of p53 and Mcl-1, compatible with survival and proliferation. Aberrant oncogene activation leads to ARF induction and inhibition of ARF-BP1/Mule activity toward p53 in the nucleus, thereby facilitating apoptosis (see Figure 1). In the cytoplasm, where ARF is not abundant, oncogene activation and possibly other types of stress may instead lead to ARF-BP1/Mule being targeted to Mcl-1, causing rapid Mcl-1 degradation and further promoting apoptosis. Whether and how the subcellular localization of ARF-BP1/Mule is indeed controlled in a signal-dependent manner remains to be explored.

p53 is a highly connected protein positioned in the center of an intricate network of feedback loops and signaling cascades (Oren, 2003). It therefore would not be surprising if p53 is found capable of modulating the outcome of ARF-BP1/Mule action, rather than merely serving as its obedient ubiquitylation target. In fact, recent work suggests that this may well be the case. Thus, the cancer-associated protein TCTP (translationally controlled tumor protein) binds to Mcl-1 and prevents its ubiquitylation and degradation (Liu et al., 2005). Although the underlying mechanism remains to be elucidated, it is not unlikely that TCTP may shield Mcl-1 from ARF-BP1/Mule or even interact physically with this E3 ligase to prevent it from promoting Mcl-1 degradation. Interestingly, TCTP expression is negatively regulated by p53 (Cans et al., 2003). It is tempting to speculate that, during a vigorous p53 response, p53-mediated downregulation of TCTP enables ARF-BP1/Mule to preferentially target Mcl-1 while letting go of p53. This is predicted to set the stage for efficient apoptosis. Notably, it is unlikely that ARF and TCTP are the only regulators of ARF-BP1/Mule activity, and the elucidation of additional regulators cannot be far off.

What is the point of having a single E3 ligase that targets both a positive (p53) and a negative (Mcl-1) regulator of apoptosis? First, it should be noted that this functional categorization is not as tight as it appears. For instance, there also exists a shorter form of Mcl-1, Mcl-1S, that is generated by alternative splicing (Michels et al., 2005). Mcl-1S retains the BH3 domain and thus should be able to bind to ARF-BP1/Mule. However, unlike full-length Mcl-1, Mcl-1S actually promotes apoptosis. Hence, downregulation of Mcl-1S is expected to increase survival. Conversely, p53 sometimes can actually exert antiapoptotic effects, particularly in the absence of acute stress. This may occur, for instance, via p53-mediated maintenance of basal levels of p21 (Oren, 2003). Nevertheless, it does remain true that p53 and Mcl-1 often have opposing effects on cell survival. It is plausible that, under most physiological

conditions, ARF-BP1/Mule does not downregulate p53 and Mcl-1 simultaneously. The biological and molecular principles that guide the selective choice of targets by ARF-BP1 may therefore hold the key to the enigma.

Owing to its ability to control the levels of key molecules such as p53 and Mcl-1, ARF-BP1/Mule is likely to be important in cell fate determination. Its large size suggests that, in addition to the HECT E3 domain and the structural motifs mediating binding of p53 and Mcl-1, ARF-BP1/Mule may hide numerous additional molecular secrets. Future work should unravel those secrets and enable the proper positioning of ARF-BP1/Mule within the highly dynamic network of signals that underpins cell fate decisions.

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Selected Reading

Cans, C., Passer, B.J., Shalak, V., Nancy-Portebois, V., Crible, V., Amzallag, N., Allanic, D., Tufino, R., Argentini, M., Moras, D., et al. (2003). Proc. Natl. Acad. Sci. USA *100*, 13892–13897.

Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005). Cell *121*, this issue, 1071–1083.

Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G.D., Dowd, P., O'Rourke, K., Koeppen, H., and Dixit, V.M. (2004). Nature *429*, 86–92.

Hershko, A., and Ciechanover, A. (1998). Annu. Rev. Biochem. *67*, 425–479.

Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Cell *112*, 779–791.

Liu, H., Peng, H.W., Cheng, Y.S., Yuan, H.S., and Yang-Yen, H.F. (2005). Mol. Cell. Biol. *25*, 3117–3126.

Lowe, S.W., and Sherr, C.J. (2003). Curr. Opin. Genet. Dev. *13*, 77–83.

Michels, J., Johnson, P.W., and Packham, G. (2005). Int. J. Biochem. Cell Biol. *37*, 267–271.

Oren, M. (2003). Cell Death Differ. *10*, 431–442.

Zhong, Q., Gao, W., Du, F., and Wang, X. (2005). Cell *121*, this issue, 1085–1095.

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